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Novel Pyrazole Cannabinoids: Insights into CB_1 Receptor Recognition and Activation

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ABSTRACT

Synthesis of an antagonist, SR141716A, that selectively binds to brain cannabinoid (CB_1) receptors without producing cannabimimetic activity in vivo, suggests that recognition and activation of cannabinoid receptors are separable events. In the present study, a series of SR141716A analogs were synthesized and were tested for CB_1 binding affinity and in a battery of in vivo tests, including hypomobility, antinociception, and hypothermia in mice. These analogs retained the central pyrazole structure of SR141716A with replacement of the 1-, 3-, 4-, and/or 5-substituents by alkyl side chains or other substituents known to impart potent agonist activity in traditional tricyclic cannabinoid compounds. Although none of the analogs alone produced the profile of cannabimimetic effects seen with full agonists, several of the 3-substituent analogs with higher binding affinities showed partial agonism for one or more measures. Cannabimimetic activity was most noted when the 3-substituent of SR141716A was replaced with an alkyl amide or ketone group. None of the 3-substituted analogs produced antagonist effects when tested in combination with 3 mg/kg Δ^9-tetrahydrocannabinol (Δ^9-THC). In contrast, antagonism of Δ^9-THC's effects without accompanying agonist or partial agonist effects was observed with substitutions at positions 1, 4, and 5. These results suggest that the structural properties of 1- and 5-substituents are primarily responsible for the antagonist activity of SR141716A.

Compounds that bind to brain cannabinoid (CB_1) receptors show a large degree of diversity in chemical structure and include classical tricyclic and bicyclic cannabinoids, aminoalkylindoles, indoles, pyrroles, and anandamides. Each of these classes of compounds shares a similar profile of pharmacological activity in vivo with the prototypic tricyclic cannabinoid, Δ^9-tetrahydrocannabinol (Δ^9-THC), albeit they differ in potency and there are some differences in efficacy in individual assays (Compton et al., 1992, 1993; Adams et al., 1995; Wiley et al., 1998). These in vivo cannabimimetic effects include hypoactivity, hypothermia, antinociception, and catalepsy in mice (Martin et al., 1991; Smith et al., 1994), Δ^9-THC-like discriminative stimulus effects in rats and monkeys (see Wiley, 1999 for review), and static ataxia in dogs (Lichtman et al., 1998).

With the synthesis of a CB_1 cannabinoid antagonist, SR141716A, a new class of cannabinoids was revealed (Rinaldi-Carmona et al., 1994). SR141716A selectively binds to cannabinoid CB_1 receptors without producing cannabimimetic activity in vivo (Compton et al., 1996), suggesting that binding and activation of cannabinoid receptors are separable events. Consequently, structure-activity relationship (SAR) studies with analogs of this antagonist provide a unique opportunity to compare the structural requirements for binding and antagonist activity to those required for binding and agonist efficacy. To date, only a couple of studies have been published, which systematically examined the SAR of cannabinoid CB_1 antagonists (Thomas et al., 1998; Lan et al., 1999). Although both of these studies reported CB_1 binding values for SR141716A analogs, neither involved measurement of in vivo activity of the compound alone *and* in combination with an active cannabinoid.

The purpose of the present study was synthesis of a series of analogs of SR141716A and subsequent in vitro and in vivo testing. These analogs retained a central pyrazole structure with manipulation of one of four other areas of the molecule: 1) substitution for carboxyamide and/or piperidine substituent (3-substituent substitution); 2) substitution for the 2,4-

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Manuscript results were previously reported in abstract form (Wiley et al., 2000).

ABBREVIATIONS: CB_1 receptor, brain cannabinoid receptor; anandamide, arachidonylethanolamide; CP 55,940, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; GTPγS, guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate; MPE, maximal possible antinociceptive effect; SAR, structure-activity relationship; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; Δ^9-THC, Δ^9-tetrahydrocannabinol.

dichlorophenyl group (1-substituent substitution); 3) substitution for chlorophenyl group (5-substituent substitution); or 4) substitution for the methyl (4-substituent substitution) (Fig. 1). Cannabinoid receptor binding affinities were determined then followed by in vivo testing in mice. Selected compounds with binding affinity (K_i) less than 100 nM were further tested in combination with active dose(s) of Δ^9-THC to evaluate potential antagonist effects.

Materials and Methods

Subjects. Male ICR mice (25–32 g), purchased from Harlan (Dublin, VA), were housed in groups of five. All animals were kept in a temperature-controlled (20–22°C) environment with a 12-h light/dark cycle (lights on at 7:00 AM). Separate mice were used for testing each drug dose in the in vivo behavioral procedures. The mice were maintained on a 14:10-h light:dark cycle and received food and water ad libitum. Brain tissue for binding studies was obtained from male Sprague-Dawley rats (150–200 g) purchased from Harlan Laboratories (Dublin, VA).

Apparatus. Measurement of spontaneous activity in mice occurred in standard activity chambers interfaced with a Digiscan Animal Activity Monitor (Omnitech Electronics, Inc., Columbus, OH). A standard tail-flick apparatus [described by Dewey et al. (1970)] and a digital thermometer (Fisher Scientific, Pittsburgh, PA) were used to measure antinociception and rectal temperature, respectively.

Drugs. Δ^9-THC (National Institute on Drug Abuse, Rockville, MD) and CP 55,940 (Pfizer, Groton, CT) were suspended in a vehicle of absolute ethanol, Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ), and saline in a ratio of 1:1:18. SR141716A (National Institute on Drug Abuse) and novel pyrazole cannabinoids (synthesized in our laboratories) were also mixed in 1:1:18 vehicle. All drugs were administered to the mice intravenously in the tail vein at a volume of 0.1 ml/10 g.

Membrane Preparation and Binding. The methods used for tissue preparation and binding have been described previously (Compton et al., 1993) and are similar to those described by Devane et al. (1988). All assays, as described briefly below, were performed in triplicate, and the results represent the combined data from three to six individual experiments.

Following decapitation and rapid removal of the brain, whole brain was homogenized and centrifuged. The resulting pellet was termed P_1. The supernatant was saved and combined with the two subsequent supernatants obtained from washing of the P_1 pellet. The combined supernatant fractions were centrifuged, resulting in the P_2 pellet. After further incubation and centrifuging, this pellet was resuspended in assay buffer to a protein concentration of approximately 2 mg/ml. The membrane preparation was quickly frozen in a bath solution of dry ice and 2-methylbutane (Sigma Chemical Co., St. Louis, MO) and then stored at −80°C for no more than 2 weeks. Before performing a binding assay an aliquot of frozen membrane was rapidly thawed and protein values were determined by the method of Bradford (1976).

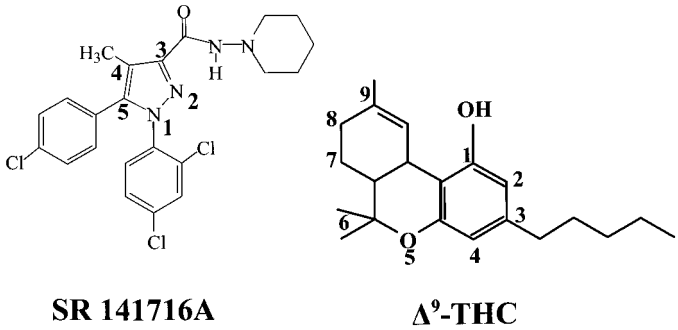


Fig. 1. Chemical structure of SR141716A with points of substituent attachment marked by numbers surrounding the pyrazole core: 1 (dichlorophenyl group), 2 (hydrogen), 3 (carboxyamide and piperidine groups), 4 (methyl group), and 5 (chlorophenyl group). For comparison purposes, the chemical structure of Δ^9-THC is also presented.

Binding was initiated by the addition of 150 μg of P_2 membrane to test tubes containing 1 nM [^{3}H]CP 55,940 (79 Ci/mmol) and a sufficient quantity of buffer to bring the total incubation volume to 1 ml. Nonspecific binding was determined by the addition of 1 μM unlabeled CP 55,940. Following incubation at 30°C for 1 h, binding was terminated by addition of ice-cold buffer and vacuum filtration through pretreated filters in a 12-well sampling manifold (Millipore, Bedford, MA). After washing, filters were placed into plastic scintillation vials (Packard, Downer Grove, IL) and shaken. The quantity of radioactivity present was determined by liquid scintillation spectrometry.

Procedure. Before testing in the behavioral procedures, mice were acclimated to the experimental setting (ambient temperature 22–24°C) overnight. Preinjection control values were determined for rectal temperature and tail-flick latency (in seconds). For agonism tests, mice were injected intravenously with drug or vehicle and, 5 min later, were placed in individual activity chambers where spontaneous activity was measured for 10 min. Activity was measured as total number of interruptions of 16 photocell beams per chamber during the 10-min test and was expressed as percentage inhibition of activity of the vehicle group. Tail-flick latency was measured at 20 min postinjection. A maximum latency of 10 s was used. Antinociception was calculated as percentage of maximum possible effect {%MPE = [(test − control latency)/(10 − control)] × 100}. Control latencies typically ranged from 1.5 to 4.0 s. At 30 min postinjection, rectal temperature was measured. This value was expressed as the difference between control temperature (before injection) and temperatures following drug administration (Δ°C). Different mice (n = 5–6) were tested for each dose of each compound. Each mouse was tested in each of the three procedures. Antagonism tests were conducted using an identical procedure with the exception that the antagonist analog was injected 10 min before the injection of 3 mg/kg Δ^9-THC.

Data Analysis. Based on data obtained from numerous previous studies with cannabinoids, maximal cannabinoid effects in each procedure were estimated as follows: 90% inhibition of spontaneous activity, 100% MPE in the tail-flick procedure, and −6°C change in rectal temperature. ED_{50} values were defined as the dose at which half-maximal effect occurred. For drugs that produced one or more cannabinoid effect, ED_{50} values were calculated separately using least-squares linear regression on the linear part of the dose-effect curve for each in vivo measure, plotted against $\log_{10}$ transformation of the dose. For the purposes of potency comparison, potencies were expressed as millimoles per kilogram. Data collected during combination tests (analog dose + 3 mg/kg Δ^9-THC) were converted to percentage antagonism [(mean score of group that received vehicle and 3 mg/kg Δ^9-THC − score obtained with analog dose and 3 mg/kg Δ^9-THC)/(mean score of group that received vehicle and 3 mg/kg Δ^9-THC) × 100]. When the resulting values showed dose-responsiveness, AD_{50} values were calculated separately using least-squares linear regression on the linear part of the percentage antagonism curve for each in vivo measure, plotted against $\log_{10}$ transformation of the dose. For the purposes of potency comparison, antagonist potencies were expressed as millimoles per kilogram.

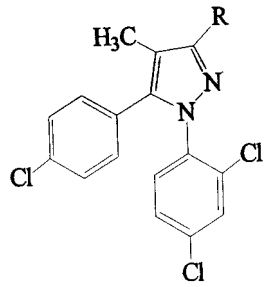
Results

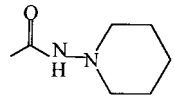
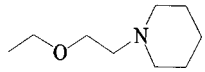
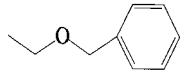
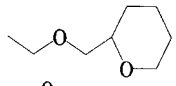
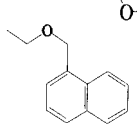
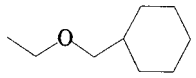
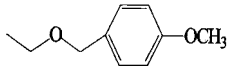
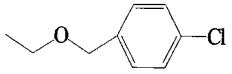
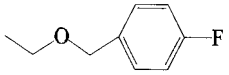
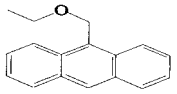
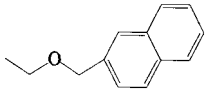
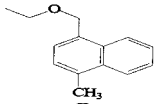
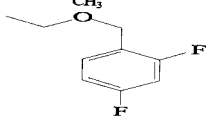
Binding Affinities. Table 1 shows binding affinities for pyrazole analogs in which the carboxyamide group of the 3-substituent of SR141716A was replaced with an alkylether group. Substitution of an alkylether group for the carboxyamide group with retention of the terminal piperidine group,

TABLE 1

Pharmacological effects of 1-(2,4-dichlorophenyl)-4-methyl-5-(4-chlorophenyl)-1*H*-pyrazoles with cyclic 3-substituents

For Tables 1–4: # indicates the maximal effect that was produced by the analog and the dose (mg/kg) at which it occurred in parentheses. ">dose" indicates that 50% activity was not achieved at this dose (mg/kg), which was the highest dose of the compound that was tested. All ED_{50} values are expressed as μmol/kg (with 95% confidence limits in parentheses).



Compound	R	K_i (nM)	SA	TF	RT
Δ^9-THC[a]		41	0.92	2.7	2.5
SR141716A		6.2[b]	>30[c]	>30[c]	>30[c]
O-848		2450 ± 720	69% (10)[#]	N.T.	N.T.
O-849		108 ± 9	>30	>30	−4 (30)[#]
O-850		351 ± 34	>10	N.T.	N.T.
O-852		78 ± 10	>10	>10	>10
O-853		388 ± 32	N.T.	N.T.	N.T.
O-869		194 ± 11	>30	>30	>30
O-870		109 ± 9	66% (30)[#]	>30	−3 (30)[#]
O-889		54 ± 2	20 (17–25)	67% (30)[#]	40 (31–52)
O-890		379 ± 38	>10	>10	>10
O-909		216 ± 29	>30	>30	>30
O-910		143 ± 15	>30	>30	>30
O-1043		53 ± 9	>30	>30	>30

SA, suppression of spontaneous activity; MPE, % maximum possible antinociceptive effect in tail-flick assay; RT, rectal temperature; N.T., not tested.

[a] From Wiley et al. (1998).

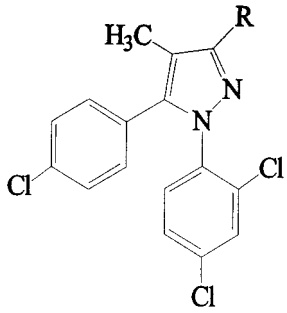
[b] From Thomas et al. (1998).

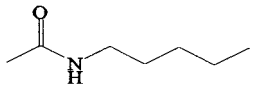
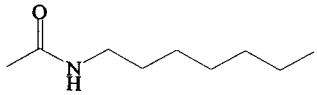
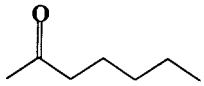
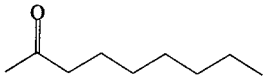
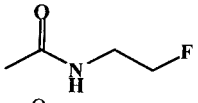
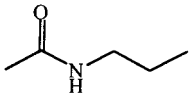
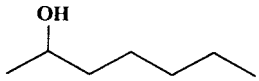
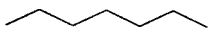
[c] From Compton et al. (1996).

as in O-848, greatly decreased binding affinity for CB_1 receptors. Although affinity was improved (compared with O-848) by substitution of various cyclic, bicyclic, or tricyclic structures for the piperidine ring of O-848, most compounds listed in Table 1 still had relatively little affinity for the CB_1 receptor (K_i > 100 nM). Notable exceptions were O-852, O-889, and O-1043, each of which had CB_1 affinity <100 nM. In addition to substitution of an alkylether for the carboxyamide at position 3 on the pyrazole core (as with all compounds in this series), these compounds had substitutions of naphthalene (O-852), 4-fluorophenyl (O-889), and 2,4-difluorophenyl (O-1043) groups for the piperidine of the parent compound, SR141716A. Nevertheless, the CB_1 affinities of these three compounds were substantially less than that of SR141716A.

Table 2 shows the binding affinities of compounds in which the *n*-piperidine at the 3-position of SR141716A was replaced by a carbon chain that more directly corresponds to the lipophilic side chain of Δ^9-THC. Some of these compounds retained the amide group at position 3 on the pyrazole core (O-1269, O-1270, O-1398, and O-1399), whereas others had a ketone substitution at this position (O-1271 and O-1272). Still others had substitutions of a heptyl chain without branching (O-1877) or with an attached 1′-hydroxyl group (O-1876). In all cases, binding affinities were greatly enhanced compared with 3-substituent substitution with an alkylether group, as in O-848, but still were 5- to 137-fold less than SR141716A. Of the compounds retaining the amide group, the *n*-pentyl compound, O-1269, had the greatest affinity. Affinity was only slightly decreased by substituting *n*-heptyl (O-1270) but was decreased 5-fold through *n*-propyl substitution (O-1399). Fluoroethyl substitution (O-1398) produced a further 5-fold reduction in affinity. Replacement of the amide group with a ketone (O-1271 and O-1272) also resulted in decreased affinity compared with compounds with identical side chain lengths that retained the amide

TABLE 2
Pharmacological effects of 1-(2,4-dichlorophenyl)-4-methyl-5-(4-chlorophenyl)-1*H*-pyrazoles with carbon chain 3-substituents



Compound	R	K_i (nM)	SA	TF	RT
O-1269		32 ± 5	11 (7–19)	21* (9–49)	11 (8–16)
O-1270		48 ± 12	27 (19–44)	20 (9–54)	12 (10–15)
O-1271		82 ± 10	>30	>30	>30
O-1272		221 ± 36	54% (30)#	>30	−3 (30)#
O-1398		852 ± 175	7* (not calculated)	13 (10–18)	8 (5–12)
O-1399		167 ± 32	9 (5–17)	24 (16–36)	10 (7–16)
O-1876		657 ± 21	>30	>30	>30
O-1877		422 ± 40	>30	>30	>30

* Estimated ED_{50} due to the fact that the dose-effect curve was not linear.

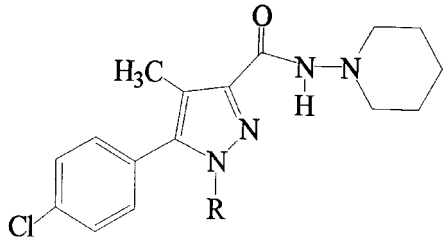
group (O-1269 and O-1270, respectively). In each of the pairs, the compound with *n*-pentyl substitution (O-1269 and O-1271) had the best affinity, suggesting that substituent length affected CB_1 receptor binding of both series.

In SR141716A, a 2,4-dichlorophenyl group is attached to the pyrazole ring at position 1. Substitutions for this 1-substituent (Table 3) decreased CB_1 receptor binding affinities. Removal of the chlorines from the 2,4-dichlorophenyl group (O-1300) decreased affinity by 24-fold compared with SR141716A. Removal of the chlorine at the 2-position of the 2,4-dichlorophenyl group and replacement of the chlorine at the 4-position with a *n*-butyl (O-1254) or *n*-pentyl (O-1255) chain further reduced affinity, with the *n*-pentyl compound having almost 2-fold less affinity than the *n*-butyl compound. In contrast, branching of the substituent at the *para*-position of the phenyl group at the 1-position of SR141716A (i.e., substitution of a *p*-secbutyl group; O-1253) increased affinity compared with the nonbranched chain analog O-1254. Nevertheless, none of the 1-substituent substitutions presented in Table 3 produced compounds with CB_1 receptor binding affinities that equaled that of SR141716A. Although the affinity of O-1253, the compound with the best affinity, was nearly equal to that of Δ^9-THC, it was 8-fold less than that of SR141716A.

Table 4 shows the affinities for analogs in which the 4- and/or 5-substituent were manipulated. Replacement of the *p*-chlorophenyl group at the 5-position of the pyrazole core with a branched carbon chain (1′-methylpentyl) resulted in the compound (O-1559) with the lowest binding affinity. Other compounds in this series retained the phenyl group of SR141716A but had methylated or nonmethylated pentyl substitution for the chloro at the *para*-position of the phenyl. *n*-Pentyl substitution (O-1302) produced a compound with CB_1 receptor binding affinity approximately 3-fold greater than that of SR141716A. Substitution of a 1′-methylpentyl chain (O-1690) did not substantially alter affinity nor did an iodine substitution for the methyl group at the 4-position of the pyrazole core (O-1704). An identical compound with a bromine substitution at the 4-position of the pyrazole only slightly increased affinity (O-1691), whereas a hydrogen substitution at this position produced a 12-fold decrease in affinity (O-1710).

Structure-Activity Relationship for Agonist Activity in Mice. 3-Substituent substitution of an alkylether group for the amide and various cyclic structures for the piperidine of SR141716A resulted in analogs that engendered slight in vivo cannabimimetic effects. Minor activity (30–70% of maximum effect) was observed with several compounds (Table 1).

TABLE 3
Pharmacological effects of *N*-(piperidin-1-yl)-5-(4-chloro-phenyl)-3-carboxamide-4-methyl-1*H*-pyrazole with various 1-substituents



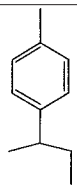
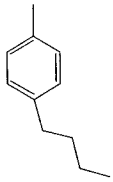
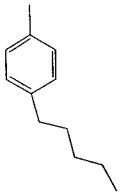
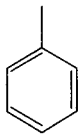
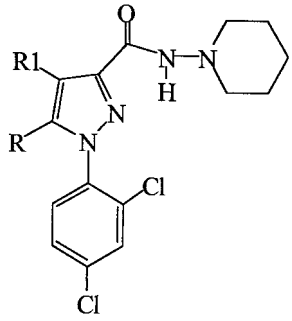
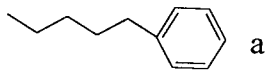
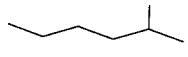
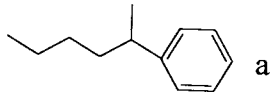
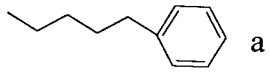
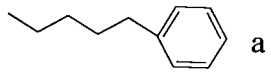
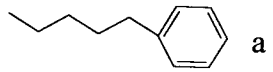
Compound	R	K_i (nM)	SA	TF	RT
O-1253		47 ± 2	>30	>30	>30
O-1254		226 ± 4	>30	>30	>30
O-1255		433 ± 103	>30	>30	>30
O-1300		150 ± 20	>30	>30	>30

TABLE 4
Pharmacological effects of *N*-(piperidin-1-yl)-1-(2,4-dichloro-phenyl)-3-carboxamide-4-methyl-1*H*-pyrazole with various 5- and 4-substituents



Compound	R	R1	K_i (nM)	SA	TF	RT
O-1302	 a	CH_3	2.1 ± 0.08	>30	>30	>30
O-1559	 a	CH_3	233 ± 3	>30	>30	>30
O-1690	 a	CH_3	2.6 ± 0.13	>30	>30	>30
O-1691	 a	Br	1.5 ± 0.22	>30	>30	>30
O-1704	 a	I	2.2 ± 0.15	>30	>30	>30
O-1710	 a	H	27 ± 0.86	>30	>30	>30

a, point of attachment to the pyrazole core at R.

The most potent cannabimimetic activity in this series was produced by a compound (O-889) with a 3-substituent substitution of a *p*-fluorophenyl methoxy group. O-889 had full or partial activity in all three assays and also had one of the highest CB_1 receptor affinities in the series (Table 1). In addition, O-889 stimulated locomotor activity by about 30% at a dose lower than those that produced suppression of locomotor activity (Table 5). O-852 also stimulated locomotion by 52%, but unlike O-889, this compound did not inhibit locomotor activity at higher doses, nor was it active in the antinociceptive or hypothermia assays.

In contrast, analogs in which a lipophilic carbon chain replaced the terminal piperidine of the 3-substituent of SR141716A showed greater cannabimimetic activity (Table 2). Retention of the amide group with substitution of *n*-pentyl or *n*-heptyl for the terminal piperidine of SR141716A (O-1269 and O-1270, respectively) resulted in agonist activity, whereas ketone substitution for the amide group with identical *n*-pentyl and *n*-heptyl substitutions for the piperidine (O-1271 and O-1272) eliminated in vivo cannabimimetic activity. Substitution of a nonbranched heptyl chain or one with a 1′-hydroxyl group also resulted in inactive compounds. In contrast, in vivo potency in all three assays was maintained or even increased when the amide group of O-1269 was shortened from *n*-pentyl to *n*-propyl (O-1399) or to 2-fluoroethyl (O-1398). Unexpectedly, however, CB_1 binding affinity decreased 5- and 27-fold (O-1399 and O-1398, respectively) with these shortened chain lengths, representing a disparity between affinity and potency for these two compounds that is not easily explained. Of this series of analogs, only O-1270 and O-1271 stimulated locomotor activity to any notable extent (29% and 54%, respectively).

Tables 3 and 4 show the results of in vivo tests for analogs with various 1-substituent or 4- and 5-substituent substitutions, respectively. None of these compounds produced any of the characteristic effects of cannabinoid agonists in the triad of tests even though each was tested up to an intravenous dose of 30 mg/kg. Indeed, several of these compounds markedly enhanced locomotor activity (50–118%) rather than inhibiting it as cannabinoid agonists do (Table 5). For analogs

TABLE 5

Maximum stimulation and inhibition of spontaneous locomotor activity by pyrazole analogs

Maximum (max) % stimulation (stim) and % inhibition (inhibit) produced by pyrazole analogs tested alone (left) and tested in combination with 3 mg/kg Δ^9-THC (right). Dose(s) (mg/kg) at which the effect occurred are given in parentheses.

Compound	Compound Alone		Compound with 3 mg/kg Δ^9-THC	
	Max Stim (Dose)	Max % Inhibit (Dose)	Max Stim (Dose)	Max % Inhibit (Dose)
3-Substituent				
O-848	None (10)	69% (10)	N.T.	N.T.
O-849	25% (10)	48% (30)	N.T.	N.T.
O-850	None (10)	43% (10)	N.T.	N.T.
O-852	52% (1)	Stim at 1, 3, and 10	N.T.	N.T.
O-853	N.T.	N.T.	N.T.	N.T.
O-869	None (30)	31% (30)	N.T.	N.T.
O-870	None (30)	46% (30)	N.T.	N.T.
O-889	31% (1)	96% (30)	None (3)	72% (3)
O-890	4% (30)	Stim at 30	N.T.	N.T.
O-909	None (30)	3% (30)	N.T.	N.T.
O-910	4% (30)	Stim at 30	N.T.	N.T.
O-1043	24% (3)	49% (30)	None (3 and 10)	88% (3)
O-1269	7% (1)	87% (30)	None (1, 3, and 10)	91% (10)
O-1270	29% (3)	80% (30)	None (10)	87% (10)
O-1271	54% (3)	7% (30)	None (3 and 10)	58% (3)
O-1272	17% (3)	54% (30)	N.T.	N.T.
O-1398	None (3, 10, and 30)	97% (10)	N.T.	N.T.
O-1399	None (3, 10, and 30)	100% (30)	N.T.	N.T.
O-1876	None (30)	11% (30)	N.T.	N.T.
O-1877	5% (30)	Stim at 30	N.T.	N.T.
1-Substituent				
O-1253	73% (30)	Stim at 1, 3, 10, and 30	50% (10)	89% (0.1)
O-1254	29% (30)	Stim at 3, 10, and 30	13% (30)	Stim at 10
O-1255	None (3, 10, and 30)	20% (10)	None (10)	65% (10)
O-1300	54% (30)	Stim at 30	N.T.	N.T.
4- and/or 5-Substituent				
O-1302	2% (10)	26% (3)	107% (30)	74% (0.3)
O-1559	118% (30)	Stim at 3, 10, and 30	N.T.	N.T.
O-1690	18% (30)	24% (10)	39% (10)	88% (0.3)
O-1691	66% (1)	15% (3)	150% (3)	91% (0.1)
O-1704	57% (1)	19% (30)	49% (10)	85% (0.3)
O-1710	4% (1)	42% (10)	None (0.3, 1, 3, and 10)	82% (10)

N.T., not tested.

with substituents at position 5, coadministration with 3 mg/kg Δ^9-THC tended to increase the degree of stimulation. As a group, these stimulatory analogs had diverse structural substitutions. Furthermore, their binding affinities ranged from 2.2 to 233 nM (O-1704 and O-1559, respectively), suggesting little correlation between CB_1 receptor affinity and potency for this effect.

In summary, with the exception of analogs with carbon side chain substitution at position 3 of the pyrazole core, strong agonist activity was not observed for any of the compounds synthesized, at least at doses below 30 mg/kg (highest dose tested for any analog). A correlation between binding affinity and agonist potency was not calculated because too few analogs were active for such a correlation to be meaningful; however, visual inspection reveals that any correlation is likely to be low. For example, although O-1269 and O-1398 have similar ED_{50} values in vivo, they have drastically different K_i values. In addition, analogs presented in Table 4 have excellent binding affinities; yet, they are not agonists in any of the in vivo assays. The influence of possible differences in pharmacokinetics among the analogs has not been evaluated.

Structure-Activity Relationship for Antagonist Activity in Mice. To assess antagonist activity, SR141716A and its analogs from each series with good binding affinities (<100 nM) were tested in combination with an active dose of Δ^9-THC (3 mg/kg i.v.). The results of these tests are presented in Table 6. As expected, SR141716A fully antagonized the suppression of locomotor activity, antinociceptive, and hypothermic effects induced by 3 mg/kg Δ^9-THC. Analogs with 3-substituent substitutions produced partial antagonism at best and often were ineffective. Maximum antagonist activity was obtained with O-1271, which produced an average of 59% antagonism across the three measures and did not have agonist properties at doses up to 30 mg/kg. With the exception of O-1253, 1-substituent substitution also did not result in marked antagonist activity. Interestingly, O-1253 also had the highest CB_1 binding affinity in this series of compounds. When tested in combination with 3 mg/kg Δ^9-THC, O-1253 produced full, dose-dependent antagonism of the antinociceptive and hypothermic effects of this dose of Δ^9-THC, but antagonized its locomotor suppressant effects only at a single dose (1 mg/kg) with stimulation at higher doses and no antagonism at lower doses.

Of all of the analogs, however, those with substitutions at position 5 (with or without concomitant substitution at position 4) produced the most consistent antagonist activity (Table 6). Retention of a phenyl group at position 5 and substitution of a pentyl or 1′-methylpentyl chain for the chloro in the *p*-chlorophenyl group of SR141716 resulted in analogs that had high CB_1 binding affinity, lacked cannabinoid agonist activity in vivo, and were potent antagonists of the antinociceptive and hypothermic effects of Δ^9-THC (Fig. 2). The phenyl group at this position appeared to be a crucial structural feature of the CB_1 binding affinity of these compounds, because O-1559, which retained a pentyl chain but

TABLE 6

Maximum percentage of antagonism or AD_{50} by pyrazole analogs

AD_{50} values expressed as μmol/kg (with 95% confidence limits in parentheses) are provided whenever % antagonism is dose-responsive. When % antagonism was not dose-responsive, values are expressed as maximum % antagonism across all doses tested. Dose(s) (mg/kg) at which the maximum antagonist effect occurred are given in parentheses. For SA, stimulation (i.e., maximum antagonism >100%) was not included in % antagonism calculations.

Compound	Dose Range Tested (mg/kg)	SA	% MPE	RT
SR141716A	0.1–3	100% (3)	96% (0.1)	0.34 (0.26–0.44)
3-Substituent				
O-852	N.T.	N.T.	N.T.	N.T.
O-889	3	2% (3)	40% (3)	11% (3)
O-1043	3 and 10	None (3, 10)	51% (10)	None (3, 10)
O-1269	1–10	38% (3)	66% (3)	43% (1)
O-1270	1–10	None (10)	37% (3)	26% (1)
O-1271	3 and 10	77% (10)	65% (10)	36% (3)
1-Substituent				
O-1253	0.1–10	97% (1)	3.3 (1.9–6.0)	4.7 (2.6–8.3)
O-1254	10 and 30	Stimulation	52% (30)	31% (10)
O-1255	10	0% (10)	13% (10)	14% (10)
5- and/or 4-Substituent				
O-1302	0.1–30	91% (1)	6.4 (2, not available)	8.6 (4.4–16.4)
O-1690	0.1–10	65% (1)	1.8 (0.6–5.3)	2.7 (1.5–4.7)
O-1691	0.1–10	6% (0.3)	1.9 (1.1–3.2)	1.8 (1.2–2.7)
O-1704	0.1–30	65% (3)	1.8 (0.7–4.3)	2.1 (1.0–4.3)
O-1710	0.3–10	50% (3)	62% (10)	51% (1)

SA, suppression of spontaneous activity; MPE, % maximum possible antinociceptive effect in tail-flick assay; RT, rectal temperature; N.T., not tested.

lacked the phenyl group, did not have good affinity. Given its low affinity, O-1559 was not tested for antagonist activity. Within the group of analogs that retained the phenyl group, differences in affinity and antagonist potency emerged. Although branching of the pentyl by addition of a 1′-methyl (O-1690) did not affect affinity compared with the nonbranched pentyl substitution (O-1302), antagonist potency was increased approximately 3-fold for antinociception and hypothermia. A bromine or iodine substitution for the methyl at position 4 effected a similar increase in potency for antagonizing Δ^9-THC-induced antinociception and hypothermia as did branching of the pentyl chain with no (iodine substitution, O-1704) or minor (bromine substitution, O-1691) increase in CB_1 binding affinity. In contrast, a hydrogen substitution at position 4 decreased affinity 10-fold and decreased maximal antagonism to 50 to 62%. Evaluation of antagonism of the locomotor suppressant effects of this series of analogs was problematic due to their prominent locomotor stimulatory effects, particularly when tested in combination with Δ^9-THC (see Table 5).

Discussion

SR141716A binds to CB_1 receptors and competitively antagonizes many of the CB_1 receptor-mediated effects of cannabinoids; hence, its structure would be expected to contain regions of overlap with those of cannabinoid agonists. An area of receptor recognition that is crucial for all known CB_1 agonists is a lipophilic side chain (e.g., THC and anandamides) or comparable moiety (e.g., nitrogen substituent of indole-derived cannabinoids) (Martin et al., 1991; Huffman et al., 1994; Thomas et al., 1996; Wiley et al., 1998). Changes in the length, branching, and flexibility of this side chain affects CB_1 receptor binding affinity and in vivo potency of cannabinoid agonists (Compton et al., 1993; Huffman et al., 1997; Martin et al., 1999). A goal of this study was to determine whether any of the pyrazole substituents of SR141716A might correspond to the C3 side chain of Δ^9-THC.

Molecular modeling suggests a possible superpositioning of

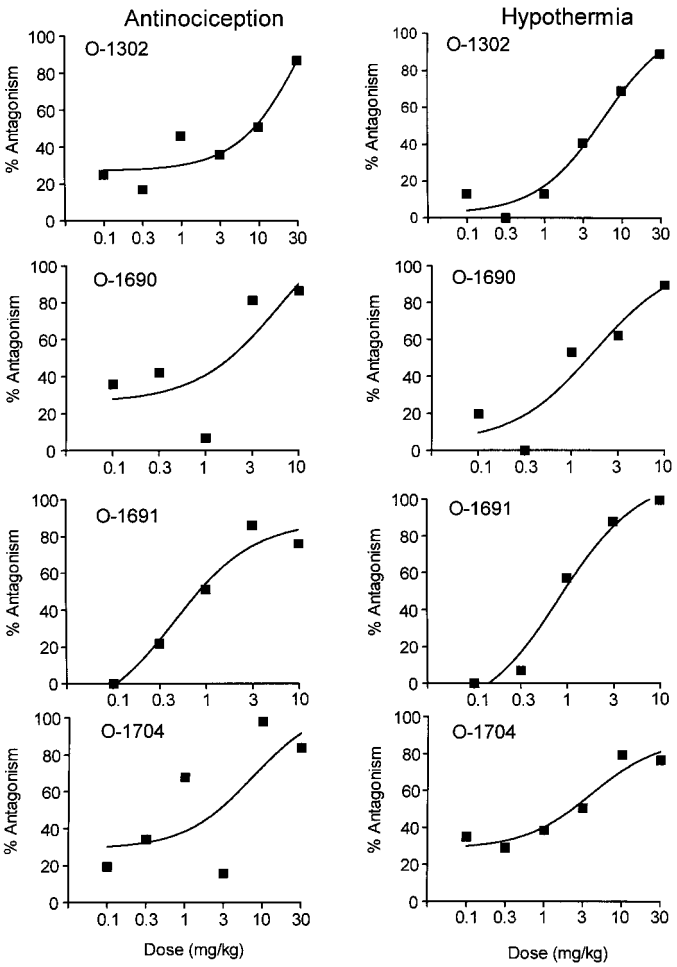


Fig. 2. Effects of 5-substituted pyrazole analogs of SR141716A on percentage antagonism of the antinociceptive (left panels) and hypothermic (right panels) effects of a 3 mg/kg (i.v.) dose of Δ^9-THC in mice. Chemical structures of each analog are presented in Table 4, and AD_{50} values are provided in Table 6.

the *para*-position of the 5-substituent in SR141716A with the pentyl side chain in Δ^9-THC (Thomas et al., 1998). Structure-activity relationships (SAR) of SR141716A analogs presented here and elsewhere (Thomas et al., 1998; Lan et al., 1999) are consistent with this proposed alignment. Retention of the phenyl group is critical for receptor affinity and antagonism, as illustrated with O-1559, which had an alkyl group at position 5 rather than a phenyl. Substitution of the *para*-portion of the phenyl substituent is also important. Deletion of the *p*-chloro group (Lan et al., 1999) greatly decreased affinity, whereas substitution of an alkyl group or an iodo/bromo (Thomas et al., 1998) enhanced affinity. Interestingly, lengthening of the pentyl side chain of Δ^8-THC (Martin et al., 1999), methylation at the first or second carbon of the chain (Huffman et al., 1997), and halogenation at the terminal end of the chain (Charalambous et al., 1991) resulted in analogs that were agonists in vivo and that had enhanced CB_1 affinity compared with the parent compound.

Although all of the *p*-pentylphenyl analogs of SR141716A (Table 4) have good affinity for CB_1 receptors, none of these analogs show cannabinoid activity in vivo. Indeed, with the exception of O-1710 (the phenyl analog with the least affinity), all are potent antagonists of the antinociceptive and hypothermic effects of Δ^9-THC. Presumably, they also will block activation of CB_1 receptors, although this hypothesis has yet to be tested in functional assays. Hence, the 5-substituent of pyrazole cannabinoids appears to be involved both in receptor recognition and in antagonism of receptor activation. Consistent with the hypothesis that this position is important for receptor recognition, Howlett et al. (2000) have shown that covalent binding of an azido or isothiocyano group to the *p*-position of the 5-phenyl ring of SR141716A irreversibly displaces [H^3]CP 55,940 from its binding site.

Consistent with the proposed overlap of the C3 side chain of Δ^9-THC and the 5-substituent of SR141716A, position 4 of the pyrazole core would correspond with either C2 or C4 of Δ^9-THC (see Fig. 1). Addition of an iodine or bromine at this position of the *p*-pentylphenyl analog of SR141716A did not substantially alter affinity, whereas hydrogen substitution (O-1710) decreased it. By comparison, halogenation of C2 of Δ^8-THC resulted in agonist analogs with decreased CB_1 affinity, and halogenation of C4 produced inactive analogs with little affinity (Martin et al., 1993). Based on these results, we suggest alignment of position 4 with C2 of Δ^9-THC; however, given the paucity of substitutions that have been made at these positions, this suggestion is tentative, pending the results of further SAR studies.

Another area likely to be involved in the antagonist actions of SR141716A is the 1-substituent. Thomas et al. (1998) have suggested that the 2,4-dichlorophenyl of SR141716A is its most unique area compared with Δ^9-THC, and that it may represent the "antagonist-conferring" region. To date, results of SAR studies support this hypothesis. Manipulation of this area by removal of one or both of the chlorine atoms (present study; Lan et al., 1999), addition of a 3-chloro or 3- or 6-iodo group (Thomas et al., 1998), or substitution of an *n*-alkyl chain for the *p*-chloro group (present study) resulted in substantial decreases in CB_1 affinity and decreased potency or loss of antagonism. Of the analogs presented here, only the branched *p*-1′-methylpropylphenyl analog (O-1253) had reasonable binding affinity and antagonist activity, although this analog still had less affinity than SR141716A and it was not as potent an antagonist. Although the positioning of the two chlorine atoms is important in determining the CB_1 affinity of these 1-substituent analogs, the presence of the 1-phenyl group is crucial for their antagonist activity. Replacement of the phenyl with an alkyl chain resulted in analogs that were partial agonists in a [^{35}S]GTPγS assay of G-protein activation (Houston et al., 1997). In contrast, analogs in which an alkyl chain was added to the phenyl group at the *p*-position showed decreased affinity and were inactive in vivo (present study). Together, these findings demonstrate that small changes in the structure in the 1-substituent result in loss of antagonism, lending support to the hypothesis that this area is important in conferring receptor recognition and antagonist activity to pyrazole cannabinoids.

The 3-substituent of the pyrazole core, the fourth area of SR141716A that was manipulated in the present study, appears to be involved in receptor recognition, as analogs that were ethers, alkyl amides, ketones, alcohol, or alkane showed greatly decreased CB_1 binding affinity. These results are in agreement with those of Lan et al. (1999). Only three of these analogs showed CB_1 binding affinity of less than 100 nM: naphthalene, 4-fluorophenyl, and 2,4-difluorophenyl substitutions. The other 3-substituent analogs that showed reasonable binding affinity were some of the alkyl amides and ketones, with the best binding affinity exhibited by the *n*-pentyl and *n*-heptyl amides and the *n*-pentyl ketone. It is noteworthy that, in each of the pairs of alkylamides and ketones, the analog with *n*-pentyl substitution had the best affinity, suggesting that substituent length affected CB_1 receptor binding in both series. Although none of the 3-substituent analogs that were tested completely blocked Δ^9-THC's effects in all assays, several were agonists or partial agonists in vivo, although most were not as efficacious as Δ^9-THC in producing the full profile of cannabimimetic effects. In addition, all of the active 3-substituent analogs were less potent than Δ^9-THC, even though some of them had approximately the same affinity for the CB_1 receptor. Together, these results suggest that the 3-substituent region is involved in receptor recognition and agonist activity.

A final issue examined in this study was the degree to which pyrazoles produce their effects through inverse agonism. SR141716A produces effects that have been considered possible indications of inverse agonism, including stimulation of locomotor activity in mice (Compton et al., 1996), inhibition of G-protein-gated inwardly rectifying potassium channels in *Xenopus* oocytes (McAllister et al., 1999), reduction in [^{35}S]GTPγS binding (Landsman et al., 1997), and increased twitch response in guinea pig ileum (Coutts et al., 2000). In the present study, substantial locomotor stimulation was observed with some analogs, particularly those with 1- and 5-substituent substitutions. Because these analogs also showed the most antagonist activity, it is tempting to speculate that this antagonism may have resulted from inverse agonism; however, several observations argue against this hypothesis. First, Δ^9-THC produces a biphasic effect on locomotor activity with stimulation at lower doses and suppression at higher doses (Evans et al., 1976). It is possible that any locomotor stimulation may be related to the agonist or partial agonist activity of some of these analogs. Second, the locomotor stimulation does not appear to be correlated with the CB_1 affinity of these analogs nor with their potency for antagonizing the in vivo effects of Δ^9-THC. For example,

the greatest degree of stimulation was produced by O-1559; yet, this analog did not have good CB_1 affinity nor was it an antagonist in vivo. These results suggest that the stimulatory effect that we observed with some of these analogs is not strongly related to action at the CB_1 receptor.

In summary, this study was undertaken to examine the pharmacological profile of various SR141716A analogs in which the 1-, 3-, 4-, and 5-positions of the pyrazole core were replaced by substituents known to impart potent agonist activity in tetrahydrocannabinols. Our results suggest that, although all three positions are important for receptor recognition, the effects of the positions differ with respect to receptor activation. The 3-position appears to be involved in agonism and receptor activation. In contrast, the 1-, 4-, and 5-positions seem to be involved in antagonism. Furthermore, the present evaluation of locomotor stimulatory effects does not support the hypothesis that the antagonist activity of pyrazole cannabinoids is related to inverse agonism. In conclusion, the present results suggest that binding and activation of the cannabinoid CB_1 receptor are separable events and that the structural properties of 1- and 5-substituents are primarily responsible for the antagonist activity of SR141716A.

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